General and Comparative Endocrinology 179 (2012) 289–295

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Strong pituitary and hypothalamic responses to photoperiod but not to 6-methoxy-2-benzoxazolinone in female common voles (*Microtus arvalis*)

Elżbieta Król [a,b,*,1], Alex Douglas [a,1], Hugues Dardente [c], Mike J. Birnie [a], Vincent van der Vinne [d], Willem G. Eijer [d], Menno P. Gerkema [d], David G. Hazlerigg [a], Roelof A. Hut [d]

[a] Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen AB24 2TZ, Scotland, UK
[b] Mammal Research Institute PAS, 17-230 Białowieża, Poland
[c] INRA UMR85, CNRS UMR7247, Université de Tours, IFCE, 37380 Nouzilly, France
[d] Chronobiology Research Unit, Center for Behaviour and Neurosciences, University of Groningen, 9747AG Groningen, The Netherlands

ARTICLE INFO

Article history:
Received 6 April 2012
Revised 25 August 2012
Accepted 2 September 2012
Available online 13 September 2012

Keywords:
6-Methoxy-2-benzoxazolinone
Melatonin
Photoperiod
Deiodinase
Pars tuberalis
Tanycyte

ABSTRACT

The annual cycle of changing day length (photoperiod) is widely used by animals to synchronise their biology to environmental seasonality. In mammals, melatonin is the key hormonal relay for the photoperiodic message, governing thyroid-stimulating hormone (TSH) production in the *pars tuberalis* (PT) of the pituitary stalk. TSH acts on neighbouring hypothalamic cells known as tanycytes, which in turn control hypothalamic function through effects on thyroid hormone (TH) signalling, mediated by changes in expression of the type II and III deiodinases (Dio2 and Dio3, respectively). Among seasonally breeding rodents, voles of the genus *Microtus* are notable for a high degree of sensitivity to nutritional and social cues, which act in concert with photoperiod to control reproductive status. In the present study, we investigated whether the TSH/Dio2/Dio3 signalling pathway of female common voles (*Microtus arvalis*) shows a similar degree of photoperiodic sensitivity to that described in other seasonal mammal species. Additionally, we sought to determine whether the plant metabolite 6-methoxy-2-benzoxazolinone (6-MBOA), described previously as promoting reproductive activation in voles, had any influence on the TSH/Dio2/Dio3 system. Our data demonstrate a high degree of photoperiodic sensitivity in this species, with no observable effects of 6-MBOA on upstream pituitary/hypothalamic gene expression. Further studies are required to characterise how photoperiodic and nutritional signals interact to modulate hypothalamic TH signalling pathways in mammals.

1. Introduction

Timing of seasonal breeding in animals has important fitness consequences, because it affects both offspring survival and the chance of survival of parents to the next breeding event. Seasonal animals use a range of environmental cues to time their reproduction with favourable food and ambient conditions. Outside the tropics, the annual change in day length (photoperiod) provides animals with the most reliable information on the time of year and forthcoming season [18,23]. In mammals, the length of day is transduced into a biochemical signal through the nightly secretion of the methoxyindole hormone, melatonin, from the pineal gland [42]. Recent work has demonstrated that melatonin acts through type 1 melatonin receptors (MT1) localised in the *pars tuberalis* (PT) of the anterior pituitary gland to control thyroid hormone action in the hypothalamus, leading to changes in seasonal reproductive function [2,19–22]. This involves the release of thyroid-stimulating hormone (TSH, a heterodimeric complex of a common glycoprotein hormone α-subunit and specific TSHβ-subunit) from the PT, at levels that increase in long photoperiod and decrease in short photoperiod, mainly due to changes in TSHβ gene expression [13]. PT-derived TSH acts on adjacent tanycytes in the ependymal cell layer lining the third ventricle of the hypothalamus to alter the expression of type II and III deiodinases (Dio2 and Dio3, respectively). The Dio2 gene encodes the enzyme that converts thyroxine (T4) into biologically active triidothyronine (T3), whereas Dio3 is responsible for degrading T4 and T3 to inactive metabolites [28]. Depending on the species, the release of TSH from PT may trigger upregulation of Dio2 expression (Soay sheep and Syrian hamster *Mesocricetus auratus*), downregulation of Dio3 expression (Siberian hamster *Phodopus sungorus*) or reciprocal regulation of both deiodinases (European hamster *Cricetus cricetus* and Fischer F344 strain rats) [2,19,20,43,45]. The balance between Dio2 and Dio3 determines local concentrations of T3 in the mediobasal hypothalamus (MBH) and hence seasonal changes in the activity of the hypothalamo-pituitary–gonadal axis.

* Corresponding author at: Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen AB24 2TZ, Scotland, UK. Fax: +44 1224 272 396.
E-mail address: e.krol@abdn.ac.uk (E. Król).
[1] These authors contributed equally to this work.

0016-6480 © 2012 Elsevier Inc. Open access under CC BY license.
http://dx.doi.org/10.1016/j.ygcen.2012.09.004

Seasonal animals are also exposed to non-photoperiodic environmental cues, which are potentially able to modify the photoperiodic signal and allow fine-tuning of breeding time to local and year-specific conditions [50]. Indeed, food, ambient temperature, rainfall and social cues have all been demonstrated to alter seasonal phenotype [38]. The reported effects of non-photoperiodic cues appear to be relatively minor, compared to the effects exerted by photoperiod. However, the reproductive impact of non-photoperiodic cues may be underestimated due to the common practise of testing animals in either long (>14 h light/day) or short (<12 h light/day) photoperiods, rather than at intermediate-duration day lengths (12–14 h light/day). Paul and colleagues [36] exposed Siberian hamsters to intermediate and long day lengths and showed that mild food restriction altered reproductive function under intermediate photoperiod, despite no effects on animals housed under long photoperiod. These results indicate that variability in food supply, particularly at vernal day lengths, may significantly modify photoperiodic induction of the reproductive axis. Despite their significance, the neuroendocrine mechanisms responsible for integration of photoperiodic and non-photoperiodic cues remain largely unknown [37].

Nutritional influences on reproduction are particularly striking in arvicoline rodents of the genus *Microtus*, which have long been of interest to ecologists because of their fluctuating population dynamics [16,26,41]. An intriguing aspect of this phenomenon is the hypothesised role of plant secondary metabolites whose dietary abundance varies seasonally. Dietary supplementation with the new growth of grasses or legumes such as alfalfa (*Medicago sativa*) is widely reported to have a marked stimulatory effect on reproduction in North American and European vole species, effectively acting as a signal for spring [5,30,31,44]. While new plant growth in general is energetically and nutritionally rich, Sanders and Berger have suggested that the reproductive stimulatory effects of new grass are specifically due to production of 6-methoxy-2-benzoxazolinone (6-MBOA) [6,46]. 6-MBOA is a breakdown product of 2, 4-dihydroxy-7-methoxy-2–11-24-benzoxin-3-(4H)-one (DIMBOA), a primary glucoside of growing monocotyledons that provides protection against pathogens and herbivores [6,15]. Grazing of grass releases enzymes that rapidly convert DIMBOA to 6-MBOA. Thus, detection of 6-MBOA in the diet has been proposed to act as a reliable signal that the growing season has begun and that food will be abundant. The initial evidence for the 6-MBOA hypothesis derived from studies in North American vole species [6,46], and was followed by a limited number of subsequent reports of effects in other rodent species, including mice and rats [7,9,33,34,47]. Despite some structural similarity with melatonin [46,53], no satisfactory mechanism for the postulated role of 6-MBOA has been forthcoming.

In this study, we first sought to determine whether the common vole (*Microtus arvalis*), an inhabitant of open grassland and farmland habitats in Europe, shows a similar photoperiodic sensitivity at the level of the pituitary and hypothalamus to that already described for photoperiodic hamsters [2,12,20,43] and ungulates [19]. Secondly, we sought to determine whether these molecular hallmarks of photoperiodic sensitivity were also responsive to 6-MBOA treatment. We tested these ideas in young female animals initially acclimated to a short photoperiod and low ambient temperature to simulate winter conditions, and then exposed to intermediate or long photoperiods.

2. Materials and methods

2.1. Animals and experimental protocol

All procedures and experimental manipulations were authorised by the Animal Experimentation Committee of the University of Groningen (DEC 6122). Common voles were obtained from a captive-bred population that was regularly supplemented with wild animals caught in the Lauwersmeer area (Netherlands, 53° 24′ N, 6° 16′ E). The breeding colony (University of Groningen) was maintained on 14 h light/24 h, (lights on 08.00 h) at an ambient temperature of 21 °C (range 20–22 °C) and 60% relative humidity (range 50–70%). All voles were housed in translucent macrolon cages (15 × 32.5 × 13 cm) provided with sawdust, dried hay and *ad libitum* water and food (standard rodent chow AM-II; Arie Blok B.V., Woerden, Netherlands). The experiment was carried out in three large temperature-controlled environmental chambers that had similar ambient temperatures and relative humidity, but different photoperiod regimes (details below).

The voles used in the experiment (48 females) were born between December 2010 and January 2011, weaned at 3 weeks of age and kept in same-sex sibling groups until housed individually. At 1–7 weeks of age, animals were exposed to a short day winter photoperiod (8 h light/24 h, lights on 08.00 h) for a total of 10 weeks. During the first 6 weeks of the acclimation period, the ambient temperature was regulated at 21 °C, after which voles were separated into individual cages while the temperature decreased gradually (over 2 weeks) to 12 °C (range 11–13 °C) and remained at this level until the end of experiment. We chose 12 °C because previous studies demonstrated that the responses of voles to photoperiodic manipulation were more pronounced at lower rather than higher ambient temperatures [27].

Following the acclimation period, voles were weighed (±0.01 g) and assigned to 6 groups (3 photoperiod regimes × 2 types of injectate) of 8 animals that were matched for mean body mass and age. These voles were then individually housed and exposed (day 0) to either spring photoperiod (12 h light/24 h, lights on 04.00 h, $n = 16$), summer photoperiod (16 h light/24 h, lights on 00.00 h, $n = 16$) or they remained in the original winter photoperiod ($n = 16$). On days 7, 8 and 9 of photoperiod manipulation, voles were injected intraperitoneally with 6-MBOA or vehicle (details below) in the middle of the light phase (summer, 07.30–8.30 h; spring, 09.30–10.30 h; winter, 11.30–12.30 h). 24 h after the last injection (day 10), all animals were weighed and decapitated to remove brains with anatomically preserved PT. Brains were snap frozen within 2–3 min of decapitation on a brass block cooled with liquid N_2, and stored at −80 °C for *in situ* hybridisation analysis. Reproductive organs were excised, cleaned of fat and connective tissue, and wet masses of paired ovaries and uterus were recorded (±0.0001 g).

2.2. Administration of 6-MBOA

The selected dose (5 μg/day) and administration of 6-MBOA was based on the maximal observed response in a dose–response experiment performed in sub-adult montane voles [46]. 6-MBOA powder (Sigma–Aldrich Company Ltd., Gillingham, UK) was dissolved in 100% ethanol (200 μg/ml) and then stored in aliquots at −20 °C. On the day of injections, the 6-MBOA stock solution was brought to room temperature and diluted 20-fold with 0.9% saline. Each vole received one 0.5 ml injection/day of this solution or of vehicle (hereafter "control") for three consecutive days.

2.3. RNA in situ hybridisation

Messenger RNA levels were quantified by radioactive *in situ* hybridisation in 20-μm coronal brain sections using techniques described previously [14,29]. Antisense riboprobes complementary to fragments of rat MT1 (GenBank accession No. AF130341, nucleotide [nt] position 1–982) [25,39] and TSHβ (GenBank accession No. M10902, nt position 106–478) [8,12], and to fragments of vole Dio2, Dio3 and the orphan G-protein coupled receptor GPR50

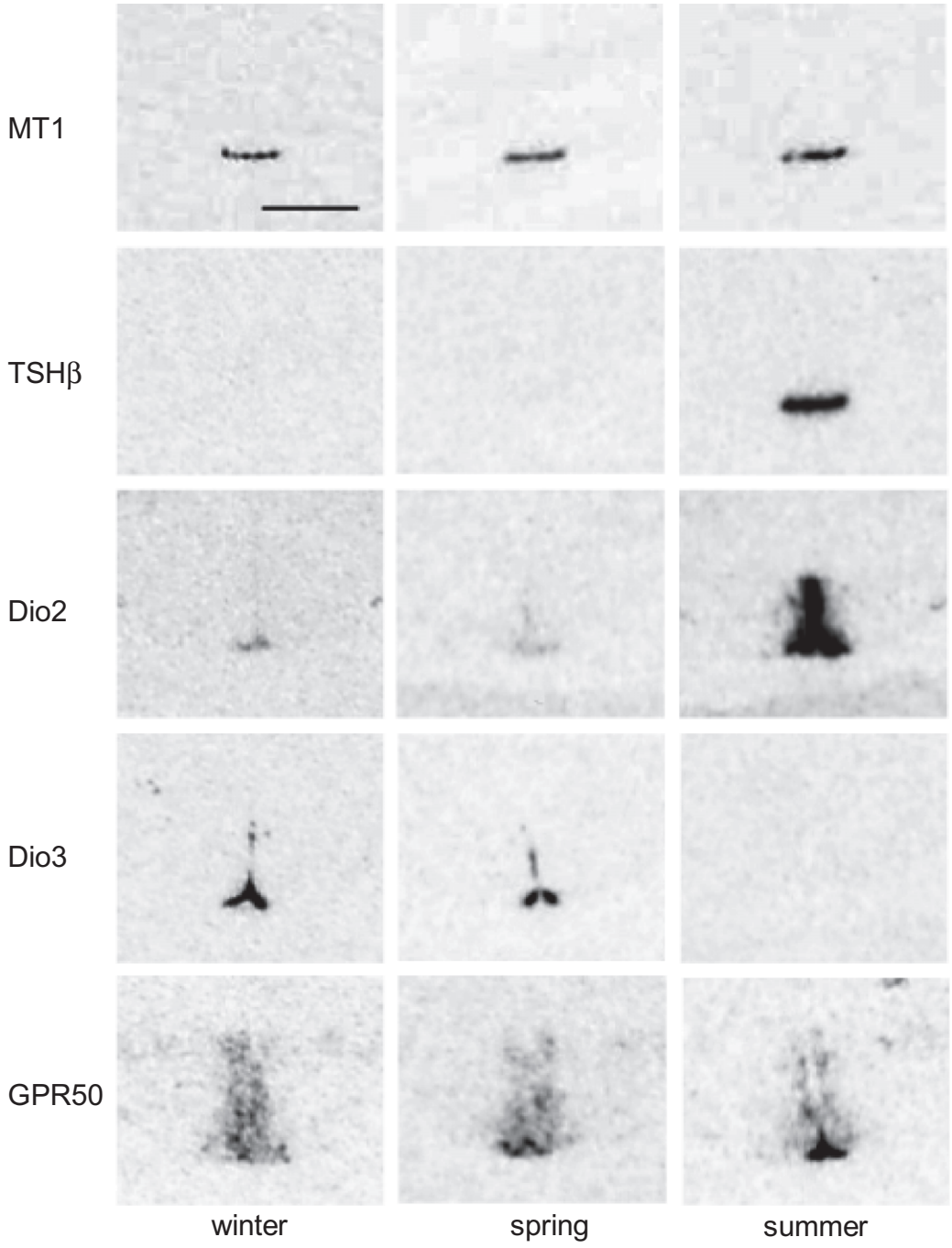

Fig. 1. Representative autoradiographs showing localisation of mRNA by *in situ* hybridisation of antisense riboprobes to adjacent coronal hypothalamic sections of a vole brain at the rostrocaudal level of the *pars tuberalis* (PT). Subadult females of the common vole were acclimated to winter photoperiod (8 h light/24 h) for 10 weeks, and then exposed to either spring photoperiod (12 h light/24 h) or summer photoperiod (16 h light/24 h) for 10 days. On days 7, 8 and 9 of photoperiod manipulation, voles were injected with 6-MBOA (images not shown) or control solution (depicted). Scale bar = 1 mm. MT1, type 1 melatonin receptor; TSHβ, thyroid-stimulating hormone β subunit; Dio2, type II deiodinase; Dio3, type III deiodinase; GPR50, G-protein coupled receptor.

(details below) were transcribed from cloned cDNA templates. The transcription reactions were performed using ^{35}S-UTP (Perkin Elmer, Boston, MA, USA). Coronal sections of vole brains were cut on a cryostat and collected throughout the rostrocaudal extent of the hypothalamus (from the optic chiasm to the mammillary bodies) onto gelatin and poly-L-lysine-coated slides, with 6–8 sections mounted on each slide. All slides for a given gene were fixed, acetylated and hybridised overnight at 58 °C with the corresponding riboprobe at approximately 1×10^6 c.p.m. per slide. The next day, slides were subjected to RNase-A digestion and stringency washes in sodium citrate buffer to remove nonspecific probe hybridisation. Slides were then dehydrated in graded ethanol solutions, air-dried and exposed to an autoradiographic film (Kodak, Rochester, NY, USA) for 3–7 days, depending on the riboprobe used. Films were digitised with an Epson Expression 1640XL scanner (Epson UK Ltd., Hemel Hempstead, UK) along with a calibrated optical density step wedge (T2115C, Stouffer Graphic Arts Equipment Co., Mishawaka, IN, USA). Autoradiographic images were quantified using ImageJ software (NIH Image, Bethesda MD, USA) that computed the integrated optical density (IOD) of the

hybridisation signal, measured above threshold and relative to a calibration curve generated from the transmission step wedge. Quantification of the signal for MT1, TSHβ, Dio2 and Dio3 was performed for all sections with detectable expression of the specific gene in the MBH. Quantification of the GPR50 signal was restricted to MBH of two adjacent sections at the rostrocaudal level of PT, which corresponded to the region with the strongest expression of Dio2/Dio3. The IOD values from specific regions of interest in all analysed sections were summed for each animal.

2.4. cDNA templates for vole Dio2, Dio3 and GPR50

Total RNA from a brain of the common vole was isolated by homogenisation in TRI Reagent (Sigma–Aldrich Company Ltd.). Reverse transcription of the total RNA into cDNA was performed using Omniscript RT Kit (Qiagen, Hilden, Germany). The fragments of vole Dio2, Dio3 and GPR50 were amplified by PCR using Platinum Taq Hifi (Invitrogen, Carlsbad, CA, USA) and subjected to electrophoresis. The PCR fragments were then extracted using QIAquick Gel Extraction Kit (Qiagen) and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). The sequence and the orientation of the PCR fragment in the vector was verified (MWG Biotech Ltd., Milton Keynes, UK) and deposited in GenBank (Dio2, accession No. JF274709, nt position 1–771; Dio3, accession No. JF274710, nt position 1–477; GPR50, accession No. HQ825084, nt position 998–1719). The resulting plasmids were linearised with appropriate restriction enzymes and used to synthesise the vole-specific riboprobes.

2.5. Statistical analysis

All data were assessed for normality and homogeneity of variance using appropriate plots of the residuals. The effects of photoperiod (winter, spring and summer) and injectate (6-MBOA and control) on PT and hypothalamic gene expression, body mass and mass of reproductive organs were determined using two-way ANOVA, with photoperiod and injectate as factors and an interaction between the factors included in the model. Because the gene expression data did not meet the required distributional assumptions, p values were calculated using permutation tests [49]. This approach is analogous to a conventional ANOVA except that the p values are obtained by pairwise permutation of the data instead of being derived from F tests. Permuted (gene expression) and Tukey HSD (body mass and reproductive organs) post hoc pairwise comparisons were used to compare individual treatment contrasts when required. All analysis was performed using the R statistical environment [40] and permutation tests were conducted using the lmPerm function [51]. An association between Dio2 and TSHβ gene expression was assessed using Pearson correlation coefficient. Statistical significance was determined at $p < 0.05$.

3. Results

3.1. PT and hypothalamic gene expression

The anatomical distribution of MT1, TSHβ, Dio2 and Dio3 mRNA expression within the MBH of the common vole (Fig. 1) is similar to that described in other photoperiodic rodents [12,20,43] as well as in the Soay sheep [19]. We observed MT1 and TSHβ gene expression only in the PT, whereas the expression of Dio2 and Dio3 was restricted to the ependymal zone lining the ventral part of the third ventricle. This region is also noted for photoperiodic expression of the orphan melatonin-related receptor GPR50 [3], which has been linked to metabolic rate in mice [4,24]. Consistent with these reports, we observed GPR50 expression in the ependymal zone as well as in a number of other hypothalamic sites (Fig. 1 and data not shown).

Densimetric analysis of gene expression is summarised in Fig. 2. The interaction between photoperiod and 6-MBOA treatment was initially included in all statistical analyses of gene expression, but was subsequently removed due to lack of statistical significance ($p > 0.42$). The level of MT1 expression in PT was not affected by photoperiod or administration of 6-MBOA (photoperiod, df = 2,44, $p = 0.11$; 6-MBOA, df = 1,44, $p = 0.88$) (Fig. 2A). The expression of

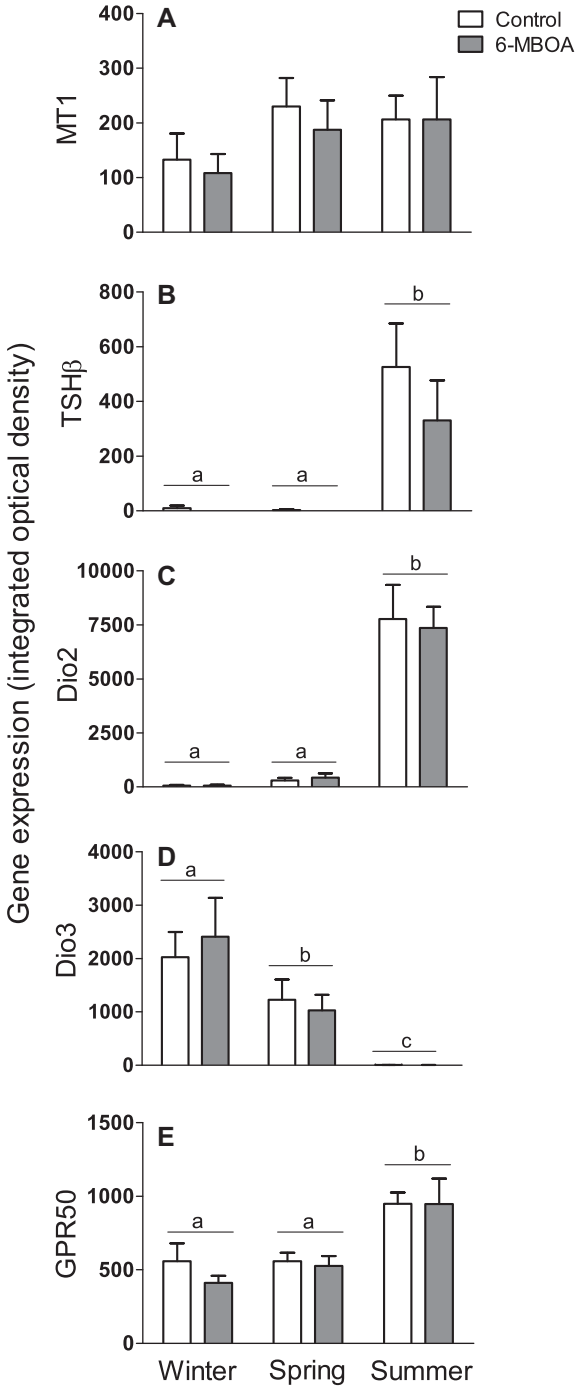

Fig. 2. Effects of photoperiod and 6-MBOA treatment on gene expression in the *pars tuberalis* (PT) and mediobasal hypothalamus (MBH) of subadult female common voles. The bar charts show integrated optical measurements of expression of MT1 (A) and TSHβ (B) in the PT, and of Dio2 (C), Dio3 (D) and GPR50 (E) in the MBH. Data are means + SEM ($n = 8$). The effects of 6-MBOA were not significant ($p > 0.28$); different letters above bars indicate significant differences between photoperiod regimes ($p < 0.04$).

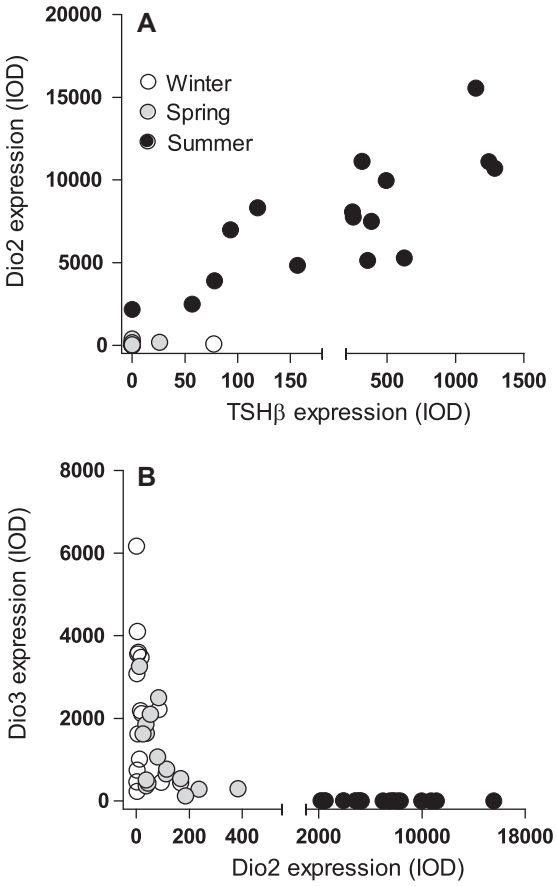

Fig. 3. Relationships between Dio2, Dio3 and TSHβ expression levels in subadult female common voles. (A) scatterplot of Dio2 *versus* TSHβ expression; (B) scatterplot of Dio3 *versus* Dio2 expression. The X-axes are split into two sections for clarity, with each section having a different scale. Each plot combines data from winter ($n = 16$), spring ($n = 16$) and summer ($n = 16$) animals. Data for 6-MBOA treated and control animals were pooled as the effects of 6-MBOA were not significant ($p > 0.28$).

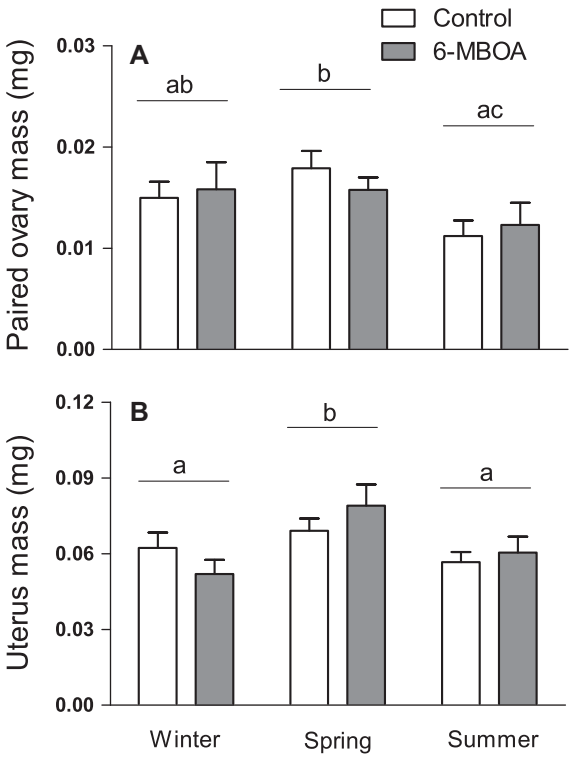

Fig. 4. Effects of photoperiod and 6-MBOA treatment on mass of paired ovaries (A) and uterus (B) in subadult female common voles. Data are means + SEM ($n = 8$). The effects of 6-MBOA were not significant ($p > 0.81$); different letters above bars indicate significant differences between photoperiod regimes ($p < 0.04$).

all other genes was highly photoperiodic (df = 2,44, $p < 0.001$ in all cases), with no significant effects of 6-MBOA (df = 1,44, $p > 0.28$ in all cases) (Fig. 2B–E). The levels of TSHβ and Dio2 expression were similarly regulated by photoperiod, with high levels in voles exposed to summer photoperiod, and minimal or undetectable levels of expression under winter or spring photoperiods (pairwise comparisons, $p < 0.001$). As a result, there was a strong positive correlation between the levels of Dio2 and TSHβ expression ($r = 0.83$, $p < 0.001$, $n = 48$) (Fig. 3A). In contrast to Dio2, the expression of Dio3 was highest in voles exposed to winter photoperiod, intermediate under spring photoperiod and undetectable under summer conditions ($p < 0.007$ for all pairwise comparisons between means). Further examination of the inverse relationship between Dio2 and Dio3 gene expression revealed that individuals tended either to express Dio2 or to express Dio3, rather than both these transcripts simultaneously (Fig. 3B). The expression level of GPR50 in the ependymal layer was higher in voles exposed to summer photoperiod than under winter or spring day lengths (pairwise comparisons, $p < 0.04$), but this effect was less dramatic than was the case for Dio2 expression in the same anatomical region.

3.2. Body mass and reproductive organs

Body and reproductive organ mass data are summarised in Fig. 4. The interaction between photoperiod and 6-MBOA treatment was initially included in all models of body and organ masses, but was subsequently removed due to lack of statistical significance ($p > 0.24$). Prior to photoperiod manipulation, voles assigned to the experimental groups did not differ in their mean body mass (photoperiod, $F_{2,44} = 0.2$, $p = 0.82$; 6-MBOA, $F_{1,44} = 0.02$, $p = 0.88$). Neither photoperiod nor administration of 6-MBOA had significant effects on vole body mass at the end of experiment (photoperiod, $F_{2,44} = 1.2$, $p = 0.30$; 6-MBOA, $F_{1,44} = 0.1$, $p = 0.74$). There were small but significant effects of photoperiod on masses of paired ovaries and uterus, with no significant effects of 6-MBOA (ovaries: photoperiod, $F_{2,44} = 4.0$, $p = 0.02$; 6-MBOA, $F_{1,44} = 0.002$, $p = 0.97$; uterus: photoperiod, $F_{2,44} = 4.9$, $p = 0.01$; 6-MBOA, $F_{1,44} = 0.06$, $p = 0.81$). Voles exposed to spring photoperiod had heavier ovaries than summer voles and heavier uterus than animals under winter or summer day lengths (pairwise comparisons, $p < 0.04$).

4. Discussion

Our study is the first to demonstrate the effects of photoperiod on the pituitary TSHβ and hypothalamic deiodinase gene expression in any member of the genus *Microtus*. In our experimental paradigm, we sought to establish a winter-like physiological state by acclimation to short photoperiod and low ambient temperature, and we predicted that this would lead to suppressed TSHβ and Dio2 expression, but elevated Dio3 expression. Our data strongly confirm these predictions, validating the basic experimental approach, and suggesting that, as in other mammalian groups, the TSH/deiodinase pathway is crucial to seasonal reproductive control. Additionally, we found that the orphan melatonin-related receptor GPR50 is photoperiodically controlled in the common vole, consistent with the emerging involvement of this gene in hypothalamic regulation of metabolic rate [4,24].

In contrast to these clear-cut effects of photoperiod, we were unable to observe any effect of the plant metabolite 6-MBOA on pituitary/hypothalamic gene expression or on downstream reproductive physiology in *M. arvalis*. This molecule has previously been linked to new growth of grasses that make up a large part of the diet of *Microtus* species, although its role in reproductive control of *M. arvalis* has not been determined. Nevertheless, the concept that this species is sensitive to springtime nutritional signals from growing plants is supported by studies comparing the effects of spring and fall harvested alfalfa (a legume in which 6-MBOA has not been detected) on ovarian activity [30].

The protocol we followed for administering 6-MBOA was carefully designed based on the work of Sanders and colleagues in North American vole species, as well as in Swiss Webster laboratory mice [46]. Based on this earlier work, we would have expected to see overt effects on reproductive organ weights within the time frame of our experiment. We therefore do not favour the possibility that the lack of observed effects was due to a failure of drug delivery. Moreover, by designing our study to include short, intermediate and long photoperiods, we sought to reveal subtle modulatory effects potentially masked by strong stimulatory or inhibitory day lengths. The intermediate spring photoperiod clearly generated an intermediate state in terms of hypothalamic gene expression profile (reduced Dio3 expression compared to winter animals, but a continuing absence of Dio2 expression), consistent with our experimental aims. At this moment, we speculate that while common voles share with their North American cousins a sensitivity to nutritional cues to time reproduction, 6-MBOA is not important among these cues. Importantly, responsiveness to 6-MBOA has so far been reported only for voles from the main North American clade (*M. montanus*, *M. pinetorum* and *M. ochrogaster*), while *M. arvalis* belongs to the genetically distant main European clade [17]. This leaves open the possibility that responsiveness of *Microtus* voles to 6-MBOA might be clade-specific, reflecting the evolutionary history of rapidly radiating taxa within *Microtus* [17,48].

As in our earlier work on the Soay sheep [19], we were unable to detect the expression of MT1 within the MBH of the common vole and we confirmed in this species the presence of MT1 within the PT (Fig. 1). This provides further support for the central role of the PT in relaying the melatonin photoperiodic signal [1,10,11]. Exposure of voles to summer photoperiod for 10 days generated a large increase in the expression of TSHβ in the PT, which is consistent with the uniform response of PT to long days manifested by all vertebrate species studied so far, including the Japanese quail *Coturnix japonica* [32], Soay sheep [13,19], photoperiodic rodents [20,45,52] and non-photoperiodic mice [35]. As in the European hamster and Fischer F344 strain rats [20,45], the increase in the expression of TSHβ in voles exposed to summer photoperiod was paralleled by the increased expression of Dio2, with concomitant downregulation of Dio3 transcript. The highly significant and positive correlation between the expression levels of Dio2 and TSHβ (Fig. 3A) is likely to reflect a causal relationship, since intracerebroventricular administration of TSH to short day acclimated quail [32] and Soay sheep [19] directly stimulates the expression of Dio2. Furthermore, photoperiodic induction of Dio2 expression is abolished in TSH-receptor knockout mice [35].

Although there is general acceptance that interactions between levels of Dio2 and Dio3 expression modify hypothalamic function by limiting T3 availability, the temporal relationship between the expression of these enzymes is complex and appears to vary between species. A striking feature of the present study was the absence of individuals co-expressing intermediate levels of these two genes: instead mutually exclusive expression prevailed. This result suggests that a flip–flop switch between winter (Dio3 dominated) and summer (Dio2 dominated) hypothalamic states may operate in this species. It will be interesting to explore the extent to which this switch operates in the field, and the extent to which it predicts reproductive activity.

Finally, the exposure of voles to summer photoperiod upregulated the expression of GPR50 in the ependymal region of the MBH, which is consistent with the downregulation of GPR50 reported in Siberian hamsters following transfer to short photoperiod [3]. Recent evidence from knockout mice [4,24] implicates GPR50 in leptin sensitivity and control of energy expenditure. Hence, we speculate that photoperiodic effects on GPR50 expression in the MBH may be linked to seasonal changes in metabolic physiology.

In summary, recent insights into the molecular pathways of melatonin action in mammals open up new avenues for investigating how photoperiodic and non-photoperiodic cues are integrated to synchronise breeding with optimal environmental conditions. Members of the genus *Microtus* may be particularly suited for this avenue because of their ecological diversity and well-documented sensitivity to nutritional signals. Our study in *M. arvalis* demonstrates that the core elements of the mammalian photoperiodic machinery operate as in other vertebrates, but we find no evidence for a role of the plant compound 6-MBOA in seasonal neuroendocrine function of this species.

Acknowledgments

The study was supported by the Biotechnology and Biological Sciences Research Council (Grant BB/G004463/1 to D.G.H.).

References

[1] P. Barrett, M. Bolborea, Molecular pathways involved in seasonal body weight and reproductive responses governed by melatonin, J. Pineal Res. 52 (2012) 376–388.
[2] P. Barrett, F.J. Ebling, S. Schuhler, D. Wilson, A.W. Ross, A. Warner, et al., Hypothalamic thyroid hormone catabolism acts as a gatekeeper for the seasonal control of body weight and reproduction, Endocrinology 148 (2007) 3608–3617.
[3] P. Barrett, E. Ivanova, E.S. Graham, A.W. Ross, D. Wilson, H. Plé, et al., Photoperiodic regulation of cellular retinoic acid-binding protein 1, GPR50 and nestin in tanycytes of the third ventricle ependymal layer of the Siberian hamster, J. Endocrinol. 191 (2006) 6876–6898.
[4] D.A. Bechtold, A. Sidibe, B.R. Saer, J. Li, L.E. Hand, E.A. Ivanova, et al., A role for the melatonin-related receptor GPR50 in leptin signaling, adaptive thermogenesis, and torpor, Curr. Biol. 22 (2012) 70–77.
[5] P.J. Berger, N.C. Negus, Influence of dietary supplements of fresh lettuce on ovariectomized *Microtus montanus*, J. Mammal. 55 (1974) 747–750.
[6] P.J. Berger, N.C. Negus, E.H. Sanders, P.D. Gardner, Chemical triggering of reproduction in *Microtus montanus*, Science 214 (1981) 69–70.
[7] G.M. Butterstein, M.H. Schadler, E. Lysogorski, L. Robin, S. Sipperly, A naturally-occurring plant-compound, 6-methoxybenzoxazolinone, stimulates reproductive responses in rats, Biol. Reprod. 32 (1985) 1018–1023.
[8] W.W. Chin, J.A. Muccini Jr, L. Shin, Evidence for a single rat thyrotropin-β-subunit gene: thyroidectomy increases its mRNA, Biochem. Biophys. Res. Commun. 128 (1985) 1152–1158.
[9] J.A. Cranford, J.O. Wolff, Stimulation of reproduction in *Peromyscus leucopus* and *Peromyscus maniculatus* with 6-MBOA in the field, V. J. Sci. 37 (1986) 240–247.
[10] H. Dardente, Does a melatonin-dependent circadian oscillator in the pars tuberalis drive prolactin seasonal rhythmicity?, J Neuroendocrinol. 19 (2007) 657–666.
[11] H. Dardente, Melatonin-dependent timing of seasonal reproduction by the pars tuberalis: pivotal roles for long daylengths and thyroid hormones, J. Neuroendocrinol. 24 (2012) 249–266.
[12] H. Dardente, P. Klosen, P. Pévet, M. Masson-Pévet, MT1 melatonin receptor mRNA expressing cells in the pars tuberalis of the European hamster: effect of photoperiod, J. Neuroendocrinol. 15 (2003) 778–786.
[13] H. Dardente, C.A. Wyse, M.J. Birnie, S.M. Dupré, A.S. Loudon, G.A. Lincoln, et al., A molecular switch for photoperiod responsiveness in mammals, Curr. Biol. 20 (2010) 2193–2198.
[14] S.M. Dupré, H. Dardente, M.J. Birnie, A.S. Loudon, G.A. Lincoln, D.G. Hazlerigg, Evidence for RGS4 modulation of melatonin and thyrotrophin signalling pathways in the *pars tuberalis*, J. Neuroendocrinol. 23 (2011) 725–732.
[15] W.W. Epstein, C.N. Rowsemitt, P.J. Berger, N.C. Negus, Dynamics of 6-methoxybenzoxazolinone in winter wheat: effects of photoperiod and temperature, J. Chem. Ecol. 12 (1986) 2011–2020.

[16] T. Ergon, X. Lambin, N.C. Stenseth, Life-history traits of voles in a fluctuating population respond to the immediate environment, Nature 411 (2001) 1043–1045.
[17] S. Fink, M.C. Fischer, L. Excoffier, G. Heckel, Genomic scans support repetitive continental colonization events during the rapid radiation of voles (Rodentia: *Microtus*): the utility of AFLPs versus mitochondrial and nuclear sequence markers, Syst. Biol. 59 (2010) 548–572.
[18] B.D. Goldman, Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of photoperiodic time measurement, J. Biol. Rhythms 16 (2001) 283–301.
[19] E.A. Hanon, G.A. Lincoln, J.M. Fustin, H. Dardente, M. Masson-Pévet, P.J. Morgan, et al., Ancestral TSH mechanism signals summer in a photoperiodic mammal, Curr. Biol. 18 (2008) 1147–1152.
[20] E.A. Hanon, K. Routledge, H. Dardente, M. Masson-Pévet, P.J. Morgan, D.G. Hazlerigg, Effect of photoperiod on the thyroid-stimulating hormone neuroendocrine system in the European hamster (*Cricetus cricetus*), J. Neuroendocrinol. 22 (2009) 51–55.
[21] D. Hazlerigg, A. Loudon, New insights into ancient seasonal life timers, Curr. Biol. 18 (2008) R795–R804.
[22] R.A. Hut, Photoperiodism: shall EYA compare thee to a summer's day?, Curr Biol. 21 (2011) R22–R25.
[23] R.A. Hut, D.G. Beersma, Evolution of time-keeping mechanisms: early emergence and adaptation to photoperiod, Philos. Trans. R. Soc. London B Biol. Sci. 366 (2011) 2141–2154.
[24] E.A. Ivanova, D. Bechtold, S. Dupré, J. Brennand, P. Barrett, S. Luckman, et al., Altered metabolism in the melatonin-related receptor (GPR50) knockout mouse, Am. J. Physiol. Endocrinol. Metab. 294 (2008) E176–E182.
[25] P. Klosen, C. Bienvenu, O. Demarteau, H. Dardente, H. Guerrero, P. Pévet, et al., The mt1 melatonin receptor and RORβ receptor are co-localized in specific TSH-immunoreactive cells in the *pars tuberalis* of the rat pituitary, J. Histochem. Cytochem. 50 (2002) 1647–1657.
[26] C.J. Krebs, M.S. Gaines, B.L. Keller, J.H. Myers, R.H. Tamarin, Population cycles in small rodents, Science 179 (1973) 35–41.
[27] E. Król, A. Tups, Z.A. Archer, A.W. Ross, K.M. Moar, L.M. Bell, et al., Altered expression of SOCS3 in the hypothalamic arcuate nucleus during seasonal body mass changes in the field vole, *Microtus agrestis*, J. Neuroendocrinol. 19 (2007) 83–94.
[28] R.M. Lechan, C. Fekete, Role of thyroid hormone deiodination in the hypothalamus, Thyroid 15 (2005) 883–897.
[29] G. Lincoln, S. Messager, H. Andersson, D. Hazlerigg, Temporal expression of seven clock genes in the suprachiasmatic nucleus and the pars tuberalis of the sheep: evidence for an internal coincidence timer, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 13890–13895.
[30] L. Martinet, M. Meunier, Influence des variations saisonnieres de la iuzerne sur la croissance, la mortalite et l'establissement de la maturite sexuells chez le campagnol des chams (*Microtus arvalis*), Ann. Biol. Anim. Biochim. Biophys. 9 (1969) 451–462.
[31] L.R. Meek, T.M. Lee, J.F. Gallon, Interaction of maternal photoperiod history and food type on growth and reproductive development of laboratory meadow voles (*Microtus pennsylvanicus*), Physiol. Behav. 57 (1995) 905–911.
[32] N. Nakao, H. Ono, T. Yamamura, T. Anraku, T. Takagi, K. Higashi, et al., Thyrotrophin in the *pars tuberalis* triggers photoperiodic response, Nature 452 (2008) 317–322.
[33] R.J. Nelson, J.M.C. Blom, 6-methoxy-2-benzoxazolinone and photoperiod: prenatal and postnatal influences on reproductive development in prairie voles (*Microtus ochrogaster ochrogaster*), Can. J. Zool. 71 (1993) 776–789.
[34] R.J. Nelson, J.R. Shiber, Photoperiod affects reproductive responsiveness to 6-methoxy-2-benzoxazolinone in house mice, Biol. Reprod. 43 (1990) 586–591.
[35] H. Ono, Y. Hoshino, S. Yasuo, M. Watanabe, Y. Nakane, A. Murai, et al., Involvement of thyrotropin in photoperiodic signal transduction in mice, Proc. Natl. Acad. Sci. USA 105 (2008) 18238–18242.
[36] M.J. Paul, J. Galang, W.J. Schwartz, B.J. Prendergast, Intermediate-duration day lengths unmask reproductive responses to nonphotic environmental cues, Am. J. Physiol. Regul. Integr. Comp. Physiol. 296 (2009) R1613–R1619.
[37] M.J. Paul, L.M. Pyter, D.A. Freeman, J. Galang, B.J. Prendergast, Photic and nonphotic seasonal cues differentially engage hypothalamic kisspeptin and RFamide-related peptide mRNA expression in Siberian hamsters, J. Neuroendocrinol. 21 (2009) 1007–1014.
[38] M.J. Paul, I. Zucker, W.J. Schwartz, Tracking the seasons: the internal calendars of vertebrates, Philos. Trans. R. Soc. London B Biol. Sci. 363 (2008) 341–361.
[39] V.J. Poirel, M. Masson-Pévet, P. Pevét, F. Gauer, MT1 melatonin receptor mRNA expression exhibits a circadian variation in the rat suprachiasmatic nuclei, Brain Res. 946 (2002) 64–71.
[40] R Development Core Team, R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing, 2011, ISBN 3-900051-07-0. URL <http://www.R-project.org/>.
[41] Raptor Group RUG/RIJP, Timing of vole hunting in aerial predators, Mammal. Rev. 12 (1982) 169–181.
[42] R.J. Reiter, The melatonin rhythm: both a clock and a calendar, Experientia 49 (1993) 654–664.
[43] F.G. Revel, M. Saboureau, P. Pévet, J.D. Mikkelsen, V. Simonneaux, Melatonin regulates type 2 deiodinase gene expression in the Syrian hamster, Endocrinology 147 (2006) 4680–4687.
[44] E.F. Rissman, R.E. Johnston, Nutritional and social cues influence the onset of puberty in California voles, Physiol. Behav. 36 (1986) 343–347.
[45] A.W. Ross, G. Helfer, L. Russell, V.M. Darras, P.J. Morgan, Thyroid hormone signalling genes are regulated by photoperiod in the hypothalamus of F344 rats, PLoS ONE 6 (2011) e21351.
[46] E.H. Sanders, P.D. Gardner, P.J. Berger, N.C. Negus, 6-methoxybenzoxazolinone: a plant derivative that stimulates reproduction in *Microtus montanus*, Science 214 (1981) 67–69.
[47] M.H. Schadler, G.M. Butterstein, B.J. Faulkner, S.C. Rice, L.A. Weisinger, The plant metabolite, 6-methoxybenzoxazolinone, stimulates an increase in secretion of follicle-stimulating hormone and size of reproductive organs in *Microtus pinetorum*, Biol. Reprod. 38 (1988) 817–820.
[48] D.A. Triant, J.A. DeWoody, Accelerated molecular evolution in *Microtus* (Rodentia) as assessed via complete mitochondrial genome sequences, Genetica 128 (2006) 95–108.
[49] W.N. Venables, B.D. Ripley, Modern Applied Statistics with S, 4th ed., Springer, New York, 2002.
[50] M.E. Visser, S.P. Caro, K. van Oers, S.V. Schaper, B. Helm, Phenology, seasonal timing and circannual rhythms: towards a unified framework, Philos. Trans. R. Soc. London B Biol. Sci. 365 (2010) 3113–3127.
[51] R.E. Wheeler, lmPerm: permutation tests for linear models, 2010, R package version 1.1-2. <http://CRAN.R-project.org/package=lmPerm>.
[52] S. Yasuo, T. Yoshimura, S. Ebihara, H.W. Korf, Photoperiodic control of *TSH-β* expression in the mammalian *pars tuberalis* has different impacts on the induction and suppression of the hypothalamo-hypopysial gonadal axis, J. Neuroendocrinol. 22 (2010) 43–50.
[53] A. Yuwiler, W.D. Winters, Effects of 6-methoxy-2-benzoxazolinone on the pineal melatonin generating system, J. Pharmacol. Exp. Ther. 233 (1985) 45–50.